Identifying Peptide Ligands for Barley α-Amylase 1 Using Combinatorial Phage Display Libraries

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A synthetic combinatorial library of 15-mer peptides expressed as N-terminal fusion to pIII on the surface of filamentous bacteriophage was screened to identify specific ligands for barley α-amylase 1. Affinity selection of phages that display tight-binding peptides was accomplished by four cycles of panning, using purified α-amylase enzyme as the immobilized binding target. The amino acid sequences of the tight-binding peptides were determined by sequencing the corresponding coding region in the viral DNA genome. A total of 13 clones, randomly selected from the final enriched library, were found to have the following sequences: TRWLVYFSRPYLVAT (8 clones), PRHVFYRWFLSNPRI (4 clones), and IVRHLFLHVYPRVLM (1 clone). Binding activity of affinity-purified phage clones was confirmed by ELISA and quantitated by PFU assay. All three peptides share a common feature of an Arg residue flanked by high numbers of Tyr and Trp/Phe residues. The phage display peptide TRWLVYFSRPYLVAT showed an activity 5-fold greater than those of the other two peptides and a dissociation constant of 4.4×10^{-9} M.

Keywords: *Peptides; ligands; phage library; barley α-amylase*

The use of filamentous bacteriophage vectors to create a vast library of peptide or protein variants linked to replicating genes has proven to be a versatile tool for identifying receptor-specific and natural ligands, mapping and mimicking epitopes, developing artificial catalytic antibodies, and studying protein−DNA binding interactions (Lowman et al., 1991; Blond-Elguindi, 1993; Emanuel et al., 1996; Smith and Petrenko, 1997). In phage peptide display, foreign DNA fragments are spliced into gene III or gene VIII of filamentous phage and the peptides are expressed as N-terminal fusions to the viral coat proteins (Scott and Smith, 1990; Smith, 1993). The library consisting of phages displaying random peptides as fusion proteins is then screened and selected for those with desired activity against a target molecule. This use of in vitro biological processes is key to the effective and rapid application of combinatorial chemistry whereby a diverse pool of molecules is created, iteratively selected, mutated, and amplified, leading to the enrichment of individual variants with desired properties.

Barley α-amylase consists of two isozyme groups, the low-p*I* (α-amylase 1) and the high-p*I* (α-amylase 2) forms, encoded by separate gene families located on chromosomes 1 and 6, respectively. These two isozymes show 80% similarity in their primary structures (Rogers, 1985) but exhibit significant differences in calcium binding, pH stability, and activity on raw starch granules and soluble substrates (Bertoft et al., 1984; MacGregor and Morgan, 1986). Isozyme 1 is present in greater abundance in ungerminated barley but is less abundant in germinating barley. α-Amylase subtilisin inhibitor, which belongs to the soybean trypsin inhibitor family (Kunitz), is known to act on α-amylase 2, but not α-amylase 1, to form a ternary complex with subtilisin (Mundy et al., 1983; Vallee et al., 1994). In this study, we demonstrate the use of phage display technique to successfully isolate clones bearing peptides that bind α-amylase 1. The recombinant phages were used directly to analyze the binding affinity of phage-bearing peptides to the enzyme.

MATERIALS AND METHODS

Crude Enzymes and Reagents. Crude α-amylase preparation (2.9 units/mg of solid) used for the purification of barley α-amylase 1 (BAA1) was obtained from Sigma (St. Louis, MO). Porcine pancreatic α-amylase (PPA) from Boehringer Mannheim (Germany) was purified by affinity chromatography before use. Polyclonal antibodies for human pancreatic α-amylase were purchased from Accurate Chemical and Scientific Corp. (Westbury, NY). Sepharose 6B and horseradish peroxidase-conjugated anti-phage antibody were obtained from Pharmacia (Piscataway, NJ).

Purification of BAA1. Crude enzyme from barley malt was precipitated with 70% $(NH_4)_2SO_4$, and the isozymes were separated by ion-exchange chromatography as described in MacGregor and Morgan (1992). Active fractions were pooled and further purified by affinity chromatography using cyclohepta-amylase-substituted epoxy-Sepharose 6B according to the method of Silvanovich and Hill (1976). Protein concentration in the fractions was estimated by absorbance at 280 nm, and enzyme activity was measured using a dye-labeled starch substrate as described in Barnes and Blakeney (1974). A unit of enzyme activity was defined as the milligrams of enzymes that give a 0.001 increase in absorbance at 620 nm per milliliter of substrate (5% w/v in water) in 15 min at 37 °C. Enzyme purity was confirmed by SDS−PAGE according to the method of Laemmli (1970) and isoelectric focusing using 5% polyacrylamide and 2% ampholytes with a pH range of 3−10. Approximately 1 mg of the purified enzyme was used for generating rabbit antiserum.

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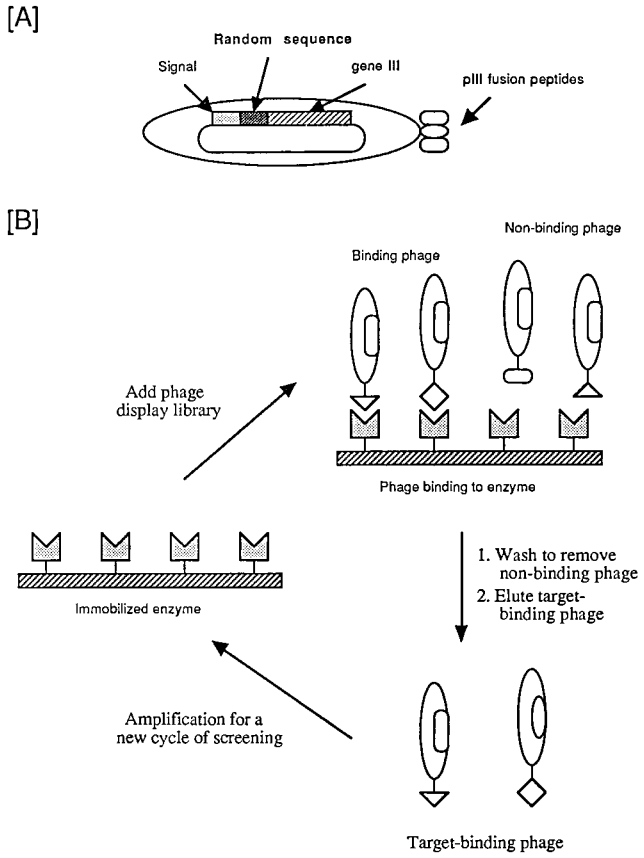

Figure 1. (A) Schematic representation of phage display type 3 vector with a single recombinant gene III bearing a foreign DNA insert. (B) Affinity selection of target binding phage from phage display library.

Affinity Selection of Peptide Ligands from a Phage Display Peptide Library. The phage display library (a gift of Prof. G. P. Smith) was constructed by inserting degenerate oligonucleotides into the *Sfi*I site downstream of the signal sequence of gene III in vector fUSE5 developed from wild-type filamentous phage fd (Scott and Smith, 1990). The degenerate coding sequence had a codon scheme of $(NNK)_{15}$, where N stands for an equal mixture of deoxynucleotides G, A, T, and C and K stands for an equal mixture of G and T. To isolate peptide ligands for BAA1, the phage peptide library was screened by four rounds of panning (Sparks et al., 1996) (Figure 1). Approximately 5 μg of BAA1 in 100 μL 0.1 M $NaHCO_3$ (pH unadjusted) was applied in triplicate to microtiter plate wells, sealed, and incubated for 1 h at 37 °C. To prevent nonspecific phage binding, 300 μL of blocking solution (5 mg of BSA/mL 0.1 M $NaHCO_3$ buffer) was next added to each well, sealed, and incubated overnight at 4 °C. The wells were washed five times with PBS/0.1% Tween 20 solution. Approximately 10^{11} PFU of library phage in 50 μL of PBS/Tween was added to each well containing the immobilized enzyme and incubated for 6 h at room temperature. Nonbinding phages were eluted by washing the wells several times with PBS/Tween. Bound phages were recovered by 10 min of incubation at room temperature with 50 μL of 50 mM glycine buffer, pH 2.0. The eluted phage was neutralized by mixing with 50 μL of 0.2 M sodium phosphate, pH 7.4, buffer in a microfuge tube. The enriched phage from this first round of panning was amplified by adding 200 μL of *Escherichia coli* K91 cell culture and incubating for 10 min, before the entire mixture was transferred to 20 mL of LB with tetracycline for overnight incubation at 37 °C. Phage supernatant was precipitated with 3 mL of 30% PEG8000/1.6 M NaCl, and the resulting phage pellet after centrifugation was resuspended in 1 mL of TBS, followed by the addition of 150 μL of PEG/NaCl. The precipitation and pelleting steps were repeated, and the phage was resuspended in 200 μL of TBS to be used for the next round of enrichment. The binding, elution, and amplification steps described above were repeated three more times before individual phage clones were analyzed.

DNA Sequencing. The enriched binding phage clones obtained from the fourth round of affinity purification were randomly picked and phage ssDNA was purified using a QIAprep Spin M13 kit (Qiagen, Santa Clarita, CA). DNA sequences encoding the inserted peptides were sequenced by the dideoxy chain termination method (Sanger et al., 1977) using a Sequenase kit supplied by Amersham (Arlington Heights, IL).

Confirmation of Binding Phage Clones by ELISA. Phage clones were picked, propagated, and purified for binding measurement by ELISA (Sparks et al., 1996). The enzyme used as binding target was immobilized onto microtiter plate wells, and bovine serum albumin was used as a negative control. For the binding reaction, 5 μL of each phage preparation in 50 μL of PBS/Tween was added to each set of wells (target enzyme and control). The microtiter plate was incubated overnight at 4 °C, and the unbound phage was removed by washing with PBS/Tween several times. The bound phage was detected using anti-phage antibody conjugated with horseradish peroxidase. The substrate for color development was 2′,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in 0.05% H_2O_2 and 50 mM sodium citrate, pH 5.0. Absorbance at 405 nm was measured using a microtiter plate scanner (Bio-Tek Instruments, Inc., Winooski, VT).

Quantitation of Binding Activity. Phage clones showing specific affinity to the target enzyme were further analyzed by PFU titering (Fang et al., 1996; Hoffman et al., 1996). Binding activity is defined as the percentage of input PFU recovered from the binding reaction between phage and immobilized enzyme in an ELISA microtiter well. The target enzyme was immobilized, blocked with BSA, and incubated with phage solution as described in the above section. Bound phage was eluted by adding 150 μL of 200 mM glycine/HCl, pH 2.0, and incubating at 25 °C for 15 min. The eluted phage was transferred to mix with 150 μL of 200 mM sodium phosphate buffer, pH 7.5. Serial dilutions were performed, and the titer was determined by infecting *E. coli* K91 and plating on NZY plates. Recovery was calculated as the total number of phage recovered following elution (in PFU) divided by the number of PFU (input) initially incubated with the immobilized enzyme.

Measurement of Dissociation Constant. The phage clone with the highest binding activity was further analyzed by measuring the dissociation constant using an indirect competition method based on ELISA as described by Friguet et al. (1985). The purified barley enzyme at various concentrations (2.9×10^{-10} to 1.2×10^{-9} M) was mixed with a constant amount of phage in PBS/Tween for 36 h until equilibrium was reached. The phage concentration used had been deduced from a calibration curve by ELISA. The free phage was determined by applying 100 mL of each mixture into the wells of a microtiter plate previously coated with α-amylase. The dissociation constant was calculated from the slope of a Klotz plot of $1/v$ versus $1/a_0$, where $v = (A_0 - A)/A_0$, where A_0 = absorbance for total phage and A = absorbance for free phage.

RESULTS

The BAA1 used as the binding target in this investigation was purified to homogeneity as determined by SDS−PAGE and IEF. The library, consisting of $\sim 10^{11}$ phage recombinants, was screened by four cycles of panning against BAA1 immobilized on microtiter plates. Following the fourth round of panning, 13 randomly selected phage clones were sequenced and the peptide sequences were deduced (Figure 2). One peptide sequence, TRWLVYFSRPYLVAT, appeared in eight separate phage clones examined. Four clones had peptides with identical sequences of PRHVFYRWFLSNPRI, and

Peptide sequence	Frequency
TRWLVYFSRPYLVAT	8
PRHVFYRWFLSNPRI	4
IVRHLFLHVYPRVLM	1

Figure 2. Amino acid sequences (deduced from nucleotide sequence) of the random peptides expressed by postive phage clones.

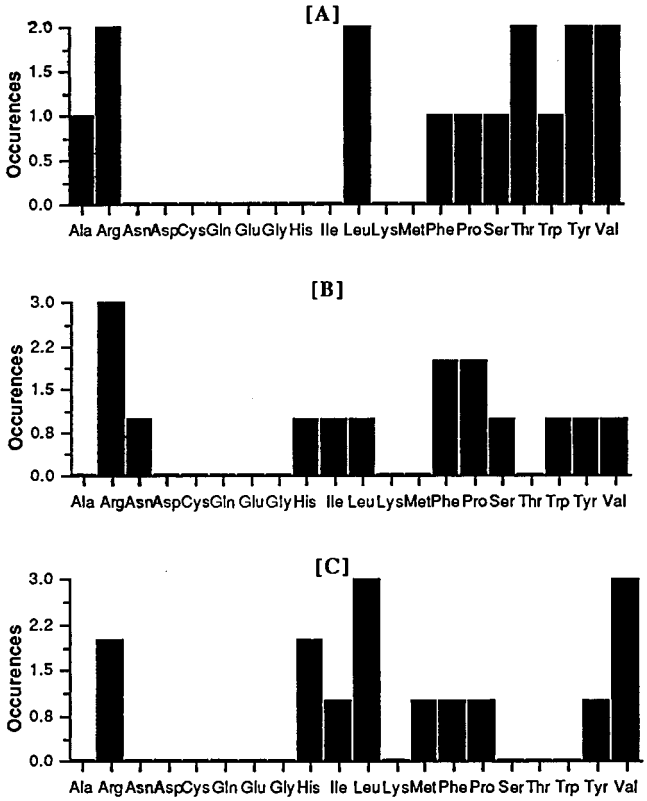

Figure 3. Histogram of amino acid composition of phage display peptides clone 1 (TRWLVYFSRPYLVAT), clone 2 (PRHVFYRWFLSNPRI), and clone 3 (IVRHLFLHVYPRVLM).

the one remaining peptide showed a sequence of IVRHLFLHVYPRVLM. These three sequences are designated clone 1, clone 2, and clone 3 peptides, respectively. The high yield of identical sequences in the isolation demonstrates the efficient enrichment of binding phage displayed peptides from a library of $\sim 10^{11}$ different 15-mer peptides. For each round of panning, phages displaying peptides that were bound to the enzymes were preferentially selected and amplified, whereas unbound or less tightly bound phages were removed by successive washing with PBS/Tween buffer.

All three peptides identified as ligands for the barley enzyme consist of at least one Arg residue and a relatively high number of polar (Tyr, Trp) and hydrophobic amino acid residues (Phe, Val, Leu, Pro) (Figure 3). The isoelectric points of these three peptides are ≥ 10. The clone 1 peptide has considerably more hydrophobic residues (Val, Leu, Ala) than the other two peptides. The clone 2 peptide contains a unique Asn residue, which is not found in the other two peptides. Peptide 3 does not contain Trp. It is speculated the addition of a charged residue and the lack of a Trp residue in clone 2 and 3 peptides, respectively, may account for the relatively low binding activity detected

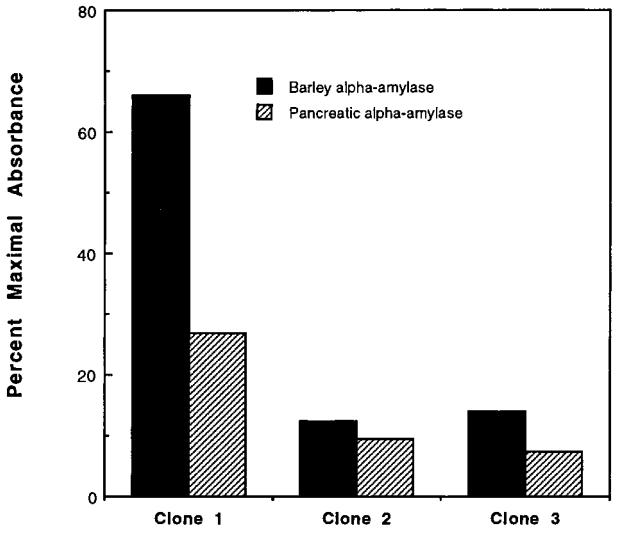

Figure 4. Confirmation of binding activity of phage clones using ELISA. Clone 1 (TRWLVYFSRPYLVAT), clone 2 (PRHVFYRWFLSNPRI), and clone 3 (IVRHLFLHVYPRVLM) were assayed for binding BAA1 and PPA.

for these two peptides in comparison to the clone 1 peptide. Peptide 2 contains a Tyr-Arg-Trp triplet reminiscent of the highly conserved segment found in all known porcine pancreatic α-amylase inhibitors (see Discussion).

The binding activity of these three phage clones was confirmed by ELISA. The phage bound to the enzyme was estimated using HRP-conjugated secondary anti-phage antibody and the colorimetric HRP substrate ASBT. The absorbance readings corresponding to the relative abundance of phage bound to the immobilized enzymes are presented in Figure 4. The clone 1 peptide, TRWLVYFSRPYLVAT, showed an absorbance ~5-fold greater than those observed for the other two peptides. Two separate experiments independently confirmed the high binding activity of this peptide. For comparison, parallel measurements were conducted using equivalent concentrations of PPA as the immobilized target. The three phage display peptides did not all show similar tight binding when tested against PPA. These results suggest that the peptides exhibit binding specificity toward the barley enzyme. The clone 1 peptide showed a significantly higher specificity than observed in the other two peptides.

For a quantitative analysis of phage binding, the percentage of input PFU retained by the immobilized target α-amylase was measured, and the percent recovery from the binding reaction is presented in Figure 5. The relative amounts of the three phage display peptides bound to BAA1 followed the same pattern as observed in the ELISA test. The results confirm that phage clone 1 indeed displayed the peptide with binding activity significantly higher (4−10-fold) than observed for the other two clones. This same peptide, when analyzed against PPA, showed less than one-fifth the binding activity. The clone 2 peptide retained only half of the activity when BAA1 was replaced by PPA as the binding target.

To further characterize the clone with the highest binding activity, an indirect competition method based on ELISA was adopted (Friguet et al., 1985). A Klotz plot of the binding of phage display peptide to α-amylase 1 is presented in Figure 6. The dissociation constant

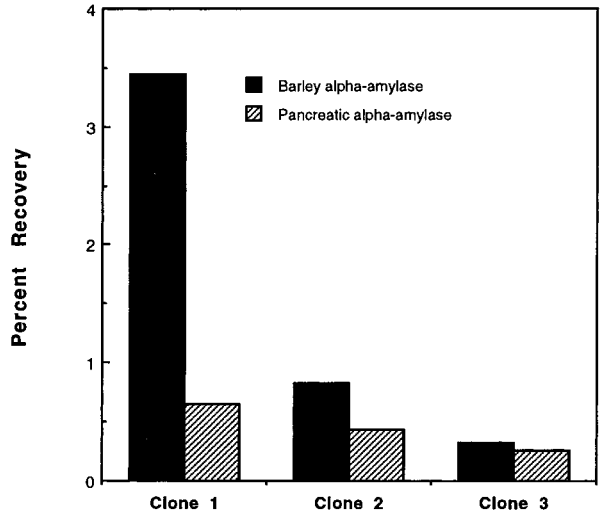

Figure 5. Quantitative analysis of binding of phage display peptides by titering. Clone 1 (TRWLVYFSRPYLVAT), clone 2 (PRHVFYRWFLSNPRI), and clone 3 (IVRHLFLHVYPRVLM) were assayed for binding to BAAI and PPA.

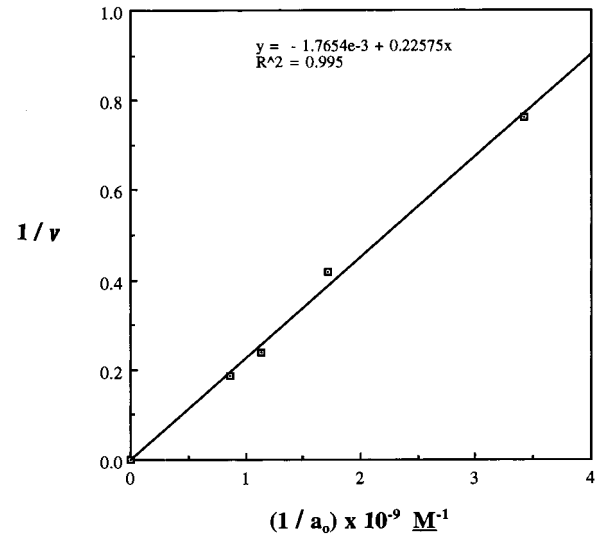

Figure 6. Klotz plot of the binding of clone 1 phage display peptide to BAA1.

of clone 1 phage display peptide calculated from linear regression of the curve was 4.4×10^{-9} M.

DISCUSSION

The present result represents the first peptide ligand ever identified for BAA1. The peptide sequences identified in this investigation have not been described previously as ligands for BAA1 or other α-amylases. A comparison with peptides identified from the same phage display peptide library using PPA as the target molecule demonstrates the high selectivity and specificity of affinity purification. In this case, a total of 16 randomly picked positive clones after the fourth round of panning were sequenced (Wong and Robertson, 1997). It is remarkable to note that the peptide sequence TRWLVYFSRPYLVAT was again identified for PPA, indicating that this peptide was active against both enzymes. However, several of the isolated phage clones shared identical sequences that were not found for the barley enzyme. The peptide ligands identified for PPA also reveal a common feature of high content of Arg, Tyr, and Trp.

The clone 1 peptide showed a binding activity for the barley α-amylase ~5-fold greater than that for the pancreatic enzyme. Although BAA1 and PPA have overall similar molecular structures, the fine details of the binding sites in the two enzymes are sufficiently different to influence the specificity for ligand binding (Kadziola et al., 1994). The number and substrate affinities of subsites at the enzyme active center vary depending on the source of the enzyme, and these differences have been attributed to the structural variations in the loops and helices forming the active sites (MacGregor, 1988). It has been reported that common bean proteinaceous αAIs inhibit the activity of mammalian and insect α-amylases, but not that of the endogenous plant enzymes (Marshall and Lauda, 1975). The enrichment of phage clones displaying different peptides for the two enzymes from the same starting library in this study implies that subtle variations exist in the ligand binding sites.

It is interesting to note that the clone 2 peptide obtained in the present investigation contains a Tyr-Arg-Trp triplet, reminiscent of a highly conserved segment in Tendamistat type inhibitors. The clone 1 peptide, which showed a 5-fold increase in the binding activity, contains Trp, Tyr, and Phe spaced between the two Arg residues. The dissociation constant was estimated to be 4.4×10^{-9} M. In comparison, an apparent dissociation constant of 0.9×10^{-6} M has been reported for BASI−barley α-amylase 2 complex (Halayko et al., 1986). In this work, the phage peptides were employed directly in a functional assay instead of using free synthetic peptides. A similar strategy has been reported for selection of trypsin inhibitors from phage display libraries (Fang et al., 1996) and for generating antibodies specific to peptide epitopes in recombinant phages (Willis et al., 1993). However, further investigation may be needed to determine the effectiveness and accuracy in the direct use of phage display peptides in replacement of free synthetic peptides.

All known proteinaceous α-amylase inhibitors from microbial sources, including Tendamistat, Haim, Paim, AI-3688, and AI-409, contain a segment of amino acids Tyr15, Trp18, Arg19, and Tyr20 (according to Tendamistat numbering) that binds to the catalytic site of PPA (Wiegand et al., 1995). In the Tendamistat−PPA complex, Arg19 forms a salt bridge with Glu233, a conserved catalytic residue in all α-amylases. The involvement of Trp, Arg, and Tyr residues has also been suggested for the inhibition of insect α-amylases by the lectin-like protein inhibitor αAI isolated from beans. The active residues identified by mutagenesis consist of Trp188, Arg74, and Tyr190 in analogy to the Trp-Arg-Tyr motif of Tendamistat (Mirkov et al., 1995). The barley α-amylase/subtilisin inhibitor (BASI) isolated from barley kernel, on the other hand, is active on barley α-amylase 2 (but not BAA1), as well as subtilisin, to form a ternary complex involving an arginine−carboxylate salt bridge (Mundy et al., 1983; Rodenburg et al., 1995). It is not unreasonable to speculate that the Arg residues found in the peptide ligands identified in this study might have the functional role of forming a salt bridge with a carboxylic side chain in the enzyme.

Binding sites outside the catalytic site have also been identified in the barley isozyme 2 as well as in pancreatic α-amylases (Kadziola et al., 1994; Hwang et al., 1997). These include a calcium binding site, a chloride binding site, and an oligosaccharide binding site. In-

vestigation of the crystal structure of isozyme 2 suggests that the sugar binding site is located at the surface of the $(\beta\alpha)_8$ domain in a region where β-cyclodextrin binds competitively with starch granules (Kadziola et al., 1994; Matsui and Svensson, 1997). Site-directed mutagenesis has confirmed that two conserved Trp residues are involved in the binding activity of this site (Sogaard et al., 1993). Screening as described in the present work could also potentially lead to identifying peptides that bind to this and other sites and not necessarily to the active site or are inhibitory. The present investigation would not allow a complete interpretation of the significance of the sequence arrangement of the identified ligands or the interactions of these peptides with a particular site. The use of computer modeling should give a better understanding on the possible binding interactions between these ligands and the enzyme.

It is remarkable to note that the use of affinity purification resulted in the isolation of ligands from phage libraries containing common features of high Arg, Trp, and Tyr reminiscent of a number of α-amylase inhibitors, considering the immobilized enzyme molecule was confronted with tens of millions of random peptides in the initial pool. This discordance is compounded by the fact that the growth rates of phage bearing different peptides may be selectively affected. For example, peptides that are rich in positively charged amino acids have been shown to be underexpressed in phage libraries (Peters et al., 1994). Thus, the selection and enrichment of peptides containing Arg are particularly suprising and indicative of the effectiveness of this technique. This study further suggests that we could selectively discriminate peptide ligands for two structurally homologous enzymes. It should also be noted that the number of recombinants (10^{11}) in the phage library, albeit a huge number, is relatively small compared to the permutation number (3×10^{19} possible peptides). The peptides we identified are not likely to include all tight-binding peptides.

The present data demonstrate that specific ligands that bind to BAA1 could be isolated from a phage display library without prior structural information. A combinatorial pool of 15-mer random peptides displayed on the surface of phage was successfully screened for individual variants with binding specificity. The enormous heterogeneous population was reduced to a small set of molecules that best fit the conformational requirement for binding by iterative selection and amplification. Theoretically, mutations could be introduced after each selection to generate a new pool of peptides isolated from the previous round of panning. This process, consisting of cycles of selection, amplification, and mutation in an in vitro system, has been successfully applied in the creation of RNA and DNA enzymes with novel properties (Tsang and Joyce, 1996). The peptide ligands identified in this study will provide a template for our subsequent studies to further enhance or modify binding and/or inhibitory activities.

ABBREVIATIONS USED

BAA1, barley α-amylase 1; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PEC, poly(ethylene glycol); PFU, plaque-forming unit; PPA, porcine pancreatic α-amylase; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline.

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